Clinical Neurology and Neurosurgery xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Clinical Neurology and Neurosurgery

journal homepage: www.elsevier.com/locate/clineuro

Immunohistochemistry analysis of bone marrow biopsies in multiple sclerosis patients undergoing autologous haematopoietic stem cells transplantation

Valentina Carrai[a,*], Irene Donnini[a], Benedetta Mazzanti[a], Renato Alterini[a], Maria Pia Amato[b], Alessandro Barilaro[b], Alberto Bosi[a], Luca Massacesi[b], Emilio Portaccio[b], Anna Maria Repice[b], Giada Rotunno[a], Riccardo Saccardi[a]

[a] Hematology Department, Careggi University Hospital, Florence, Italy
[b] Neurosciences Department, Careggi University Hospital, University of Florence, Florence, Italy

ARTICLE INFO

Article history:
Received 14 March 2012
Received in revised form 10 August 2012
Accepted 28 October 2012
Available online xxx

Keywords:
Immunohistochemistry
Bone marrow
Multiple sclerosis
AHSCT
MMP-9

ABSTRACT

Objective: Recently autologous haematopoietic stem cell transplantation (AHSCT) has been introduced for the treatment of severe forms of multiple sclerosis (MS). As little data are available on bone marrow (BM) of MS patients undergoing AHSCT, we investigated the morphological and phenotypic characteristics of MS BM.
Methods: BM biopsies of 14 MS patients screened for AHSCT and 10 control patients were evaluated to assess cellularity, morphology, immunological profile and bone marrow microenvironment. Immunohistochemistry analysis was performed to evaluate the expression of CD3, CD4, CD8, CD20, CD68, CD45, MMP-9.
Results: 8 out of 14 MS (57%) patients showed a reduction of age-related bone marrow cellularity, possibly due to previous immunosuppressive therapies. There were no differences in the T CD3+ lymphocyte expression rate amongst MS and the control patients, the CD4/CD8 ratio (2:1) was maintained as was the rate of B lymphocytes. We found an increased, although not significant, MMP-9 expression (9.2%) in the bone marrow of MS patients, when compared to the control patients (6.3%).
Conclusion: The BM of MS patients showed a reduced cellularity and CD45+ cells content in comparison to the controls. A slightly increased expression of MMP-9 was also shown, possibly confirming an involvement of this compartment in the pathogenesis of the disease.

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease believed to be mediated by autoreactive lymphocytes that invade the central nervous system (CNS) and cause oligodendrocyte, axonal and neuronal damage as well as glial scarring, resulting in demyelination, neuronal death and brain atrophy [1]. Available treatments include high-dose steroids for the management of acute phases and immunomodulatory/immunosuppressive drugs that are chronically administered to reduce the number of relapses, decrease their severity and slow down disability progression [2]. So far, no disease modifying agent has been shown to affect long-term outcome; moreover a subset of patients show an aggressive clinical pattern at the onset associated by poor response to conventional treatments. In this particular group of patients, autologous haematopoietic stem cell transplantation (AHSCT) has been recently introduced as a rescue option [3]. Clinical outcomes from most published trials have demonstrated that approximately 60% of patients show a sustained, progression- and treatment-free survival [4]. Indeed, treatment with AHSCT was associated with profound and long-lasting qualitative immunological changes [5], suggesting that beyond its immunosuppressive potential, AHSCT could have some beneficial effects also through the reconstitution of the immune system whilst avoiding the development of the autoimmune process.

The bone marrow (BM) of MS patients has been poorly studied [6,7] and, in particular, the morphological and immunohistochemical features have not been extensively assessed. A BM biopsy is routinely collected from the iliac crest before HSCT, as part of the pre-transplant clinical assessment; we therefore had the availability of BM biopsies collected in 14 MS patients enrolled in a HSCT programme at our centre. The aim of the present study was to examine with an extensive panel of morphological, cytochemical and immunohistochemical tests, the BM of MS patients at baseline. In particular, we focused on morphological features evaluated with routine haematoxylin and eosin stain (H&E stain), expression

* Corresponding author at: Hematology Department, Careggi University Hospital, Largo Brambilla 3, 50134 Florence, Italy. Tel.: +39 0557947358; fax: +39 0557947343.
E-mail address: carraiv@aou-careggi.toscana.it (V. Carrai).

0303-8467/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.clineuro.2012.10.032

of haemopoietic cells, fibres, immunological characteristics and microenvironment by immunohistochemistry and cytochemistry. We analysed the expression markers of different cellular populations (CD3, CD4, CD8, CD20, CD68, CD45) and molecules possibly involved in immunological activities of the disease, such as matrix metalloproteinase (MMP). MMPs play a crucial role as effector molecules in MS, particularly in the disruption of the blood brain barrier (BBB) by degrading basement membranes that surround blood vessels, thus favouring the trafficking of lymphocytes into CNS [8]. As several studies demonstrated that MMPs are upregulated in brain tissue, serum and cerebrospinal fluid of MS patients [9], in the current study, we also evaluated MMP-9 expression in the BM compartment.

2. Materials and methods

2.1. Patients

Fourteen patients with clinically defined MS according to the McDonald criteria [10], 12 female and 2 male, of a median age 38.5 (range 22–50), resistant to conventional therapy, were recruited for an AHSCT programme in the Haematology Unit of Careggi University Hospital (Florence, Italy). BM biopsies were performed at baseline, before the mobilisation of peripheral blood stem cells (PBSCs), from the posterior iliac crest under local anaesthesia (lidocaine) using a Jamshidi needle. Previous immunosuppressive treatments were stopped at least 1 month before the BM biopsy collection. PBSC mobilisation was carried out with 4 g/mq of cyclophosphamide followed by Filgrastim.

Five patients were diagnosed with relapsing remitting (RR) MS form and nine with secondary progressive (SP), respectively. The median EDSS at baseline was 5.5 (range 4.0–7.0). The median disease duration at baseline was 13.5 years (range 4–28), whilst the median number of relapses in the year before HSCT was 1.5 (range 0–4). Three patients showed Gadolinium positive lesions in the baseline MRI. The clinical characteristics and the previous therapies of the patients are summarised in Table 1.

Ten patients (7 female and 3 male, median age 43 years, range 34–53 years), diagnosed with non-Hodgkin lymphoma were used as controls. BM biopsy was performed at the staging phase, before any specific treatment was started. All controls were negative for lymphoma involvement of BM.

A signed informed consent form for the HSCT and accessory procedures, approved by the local ethical committee and fulfilling the Helsinki protocol, was obtained from all the patients included.

2.2. Immunohistochemical analysis and histochemistry

A formalin-fixed, paraffin-embedded section of bone marrow was deparaffinised in xylene and hydrated in graded alcohols. Antigen retroviral was performed with an EDTA buffer (pH 8.0) in the microwave, whilst MMP9 enzymatic retroviral was performed with protease 1.

To evaluate the immunological profile and bone marrow microenvironment, the following mouse monoclonal antibodies (MoAb) were used: CD3 (pre-diluted, Ventana), CD4 (1:100 dilution, Cell Marque), CD8 (pre-diluted, Ventana), CD20 (pre-diluted, Ventana), CD45/LCA (pre-diluted, Ventana), CD68/PGM1 (1:700 dilution, Dako), MMP-9 (1:100 dilution, Spring Bioscience). Immunostaining was performed with NexES® histostainer (Ventana Medical System) using a peroxidase detection kit with the 3,3′-diaminobenzidine tetrahydrochloride chromogen (iVIEW™DAB, Ventana Medical System). All staining procedures used a negative control.

The monoclonal antibodies expression was evaluated by two operators, in blind, as a percentage of positive cells over a total of eight consecutive areas at 400× magnification. The cut-off value between the low and high MMP-9 expressions was established at ≥10%.

Bone marrow fibrosis was evaluated by silver impregnation for reticulum (Gomori's silver impregnation) (Bio-Optica), following the Bauermeister classification criteria [11], as follows:

- Grade 0 = no demonstrable reticulin fibres;
- Grade 1 = occasional fine individual fibres and foci of a fine fibre network;
- Grade 2 = fine fibre network throughout most of the section; no coarse fibres;
- Grade 3 = diffuse fibre network with scattered thick coarse fibres but no mature collagen;
- Grade 4 = diffuse, often coarse fibre network with areas of collagenisation.

2.3. Statistics

Data were analysed with SPSS 10.0 for Windows and were expressed as mean ± 1 standard deviation (SD). Normal distribution of each examined parameter was verified using the Kolmogorov–Smirnoff test. Student's *t*-test for unpaired data was used to compare the differences between the two groups. A $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Morphology and fibrosis

BM cellularity in MS patients was significantly lower than in the controls, being 40.0% ± 11.0 vs 50.0% ± 3.5 ($p = 0.05$). Five patients showed severe hypoplasia, with mean cellularity 28.0% ± 8.4 that was in line with their age. Only one out of five had received three lines of immunosuppressive treatments whilst four of the five patients had received more than three lines of immunosuppressive treatments (Table 1).

The morphological characteristics of MS bone marrow were similar to the control patients' bone marrow and to that reported for normal bone marrow [12]. All haemopoietic lineages were present at each maturation stage. Myeloid precursors were localised in paratrabecular areas, whilst megakaryocytes and erythroid cells were distributed in the central areas of the BM (Fig. 1a and b).

Grade 1 bone marrow fibrosis was detected in one out of fourteen evaluable patients. No BM fibrosis was detected in the control patients.

3.2. Immunological profile and bone marrow environment

MS patients showed a mean CD45-LCA expression that was significantly lower than the control subjects (7.5% ± 4.2 vs 18.1% ± 2.7; $p = 0.001$) (Table 2), as was the cellular content (Fig. 1c and d). The rate of B lymphocytes (CD20) was normal in both groups, with a lower level in MS patients (p = ns) (Table 2).

There were no differences in the rates of CD68 (histiocyte–macrophage system) or in the T CD3+ lymphocyte expression between the MS and the control patients, which was also associated with a normal CD4/CD8 ratio (2:1) (Table 2).

The MMP9 expression in MS patients was higher than in the controls (mean expression 9.2% ± 7.0 vs 6.3% ± 2.2) (Table 2); in particular, 6 out of 14 patients (43%) exceeded the established threshold (≥10%) as opposed to in 2 out of the 10 controls (20%) ($p = 0.39$) (Fig. 1e and f). However, due to the wide dispersion of the

Please cite this article in press as: Carrai V, et al. Immunohistochemistry analysis of bone marrow biopsies in multiple sclerosis patients undergoing autologous haematopoietic stem cells transplantation. Clin Neurol Neurosurg (2012), http://dx.doi.org/10.1016/j.clineuro.2012.10.032